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Role of the phosphatidylinositol 3-kinase and extracellular signal-regulated kinase pathways in the neuroprotective effects of cilnidipine against hypoxia in a primary culture of cortical neurons

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ABSTRACT

Cilnidipine, a calcium channel blocker, has been reported to have neuroprotective effects. We investigated whether cilnidipine could protect neurons from hypoxia and explored the role of the phosphatidylinositol 3-kinase (PI3K) and extracellular signal-related kinase (ERK) pathways in the neuroprotective effect of cilnidipine. The viability of a primary culture of cortical neurons injured by hypoxia, measured by trypan blue staining and lactate dehydrogenase (LDH) assay, was dramatically restored by cilnidipine treatment. TUNEL and DAPI staining showed that cilnidipine significantly reduced apoptotic cell death induced by hypoxia. Free radical stress and calcium influx induced by hypoxia were markedly decreased by treatment with cilnidipine. Survival signaling proteins associated with the PI3K and ERK pathways were significantly increased while death signaling proteins were markedly decreased in the primary culture of cortical neurons simultaneously exposed to cilnidipine and hypoxia when compared with the neurons exposed only to hypoxia. These neuroprotective effects of cilnidipine were blocked by treatment with a PI3K inhibitor or an ERK inhibitor. These results show that cilnidipine protects primary cultured cortical neurons from hypoxia by reducing free radical stress, calcium influx, and death-related signaling proteins and by increasing survival-related proteins associated with the PI3K and ERK pathways, and that activation of those pathways plays an important role in the neuroprotective effects of cilnidipine against hypoxia. These findings suggest that cilnidipine has neuroprotective effects against hypoxia through various mechanisms, as well as a blood pressure-lowering effect, which might help to prevent ischemic stroke and reduce neuronal injury caused by ischemic stroke.

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1. Introduction

The phosphatidylinositol 3-kinase (PI3K) pathway and the extracellular signal-related kinase (ERK) pathway are known to be essential to neuronal survival (Cantley, 2002; Cantrell, 2001; Lee et al., 2008; Wruck et al., 2008). In the PI3K pathway, PI3K is activated by diverse survival factors, including insulin-like growth factor 1 and vascular endothelial growth factor, which then activates Akt (protein kinase B) (Pap and Cooper, 2002). Phosphorylated Akt directly affects its downstream targets, such as GSK-3β, BAD/Bcl-2, caspase-9, IκB kinase, and Forkhead-related transcription factor 1, which are important for neuronal survival (Lee et al., 2008; Pap and Cooper, 2002). With regard to the ERK pathway, many different stimuli including growth factors take part in its activation. Activated ERK is involved in neuronal survival and plays various roles in the activity-dependent regulation of neuronal function (Grewal et al., 1999). Therefore, activation of the PI3K and ERK pathways could be very important in neuroprotection.

Abbreviations: CCBs, calcium channel blockers; COX, cyclooxygenase; DAPI, DNA-specific fluorochrome 4′,6-diamidino-2-phenylindole dihydrochloride; DCF, 2′7′-dichlorofluorescein; DCFH-DA, dichlorodihydrofluorescein diacetate; $DCFH_2$, 2′,7′-dichlorodihydrofluorescein; DMSO, dimethyl sulfoxide; DHP, dihydropyridine; ERK, extracellular signal-related kinase; GSK-3, glycogen synthase kinase-3; HBSS, Hank's balanced salt solution; HSTF-1, heat shock transcription factor-1; IRs, immunoreactivities; LDH, lactate dehydrogenase; pAkt, phosphorylated Akt; PBS, phosphate buffered saline; pGSK-3β, phosphorylated GSK-3β; PI3K, phosphatidylinositol 3-kinase; PMSF, phenylmethylsulfonylfluoride; SCEB, sucrose-supplemented cell extract buffer; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TBS, trypan blue staining; TBST, Tris-buffered saline containing 0.05% Tween-20; TUNEL, terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling.

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Cerebral infarction is caused by a disturbance of the blood vessels in the brain due to atherothrombosis or embolism. The disturbance induces cerebral hypoxia and anoxia, resulting in a reduced supply and a complete lack of oxygen to the brain, respectively. Prolonged hypoxia induces apoptosis of neuronal cells and subsequent hypoxic brain injury (Malhotra et al., 2001; Mattiesen et al., 2009). The exact pathogenic mechanisms have not yet been established, but prolonged hypoxia affects survival signals including those from the PI3K and ERK pathways (Abdul and Butterfield, 2007; Eguchi et al., 2007), and the overreaction of several neurotransmitters, including glutamate and aspartate, due to hypoxia is involved in hypoxia-induced apoptosis. The highly elevated glutamate level caused by hypoxia results in the opening of calcium channels (Hademenos and Massoud, 1997; Rothman et al., 1987). Increased intracellular calcium activates numerous destructive enzymes, such as proteases, lipases, and endonucleases, and causes the release of cytokines and other mediators. These consecutive processes are involved in neuronal cell death and cerebral infarction (Hademenos and Massoud, 1997; Rothman et al., 1987). Therefore, there have been many studies regarding the use of calcium channel blockers (CCBs) to protect neuronal cells from hypoxia.

In spite of the numerous studies about the neuroprotective effects of CCBs, there is still much controversy surrounding their use. The neuroprotective effects of diltiazem, nifedipine, verapamil, and nimodipine, which specifically block L-type calcium channels, have been extensively studied as nifedipine was reported to exert neuroprotective effects by attenuating excitatory amino acid neurotoxicity (Weiss et al., 1990). Nimodipine was also suggested to have beneficial effects if used as an early treatment in acute ischemic stroke patients (Gelmers et al., 1988; Martinez-Vila et al., 1990). However, several other groups reported negative results regarding the effect of nimodipine in acute ischemic stroke patients (The American Nimodipine Study Group, 1992; Fogelholm et al., 2000). A recently published paper based on the results of a meta-analysis also did not find that these CCBs had a clinically important effect on ischemic stroke (Horn and Limburg, 2001).

N-type calcium channels, rather than L-type calcium channels, have recently been cited as playing important roles in neuronal cell death, and inhibitors of these channels have been suggested to possibly have neuroprotective effects (Perez-Pinzon et al., 1997; Takizawa et al., 1995; Valentino et al., 1993). Recently, CCBs able to block both N- and L-type calcium channels have been introduced. Cilnidipine, a long-acting, second-generation 1,4-dihydropyridine (DHP), is one of these CCBs and has been reported to be able to reduce infarction volume in a rat focal brain ischemia model (Hosono et al., 1992; Takahara et al., 2004; Takeda et al., 2004; Tominaga et al., 1997; Yoshimoto et al., 1991). However, the neuroprotective mechanisms of this drug have not yet been elucidated in hypoxia-injured neurons. The previous finding that omega conotoxin, a selective N-type calcium channel antagonist, protected neuronal cells against hypoxic neurodegeneration (Pringle et al., 1996) led us to hypothesize that cilnidipine-induced inhibition of N-type calcium channels might reduce hypoxia-induced neuronal cell damage.

Hypoxia is also known to increase the production of H_2O_2, one of the most well-known oxidative stresses (Zitta et al., 2010), and hypertension, one of the most important risk factors of cerebral infarction, induces oxidative stress (de Champlain et al., 2004). Oxidative stress is a common pathogenic mechanism in neuronal cell death. Therefore, there has been an enormous effort to establish therapeutic and dietary strategies to prevent oxidative stress-induced damage in the central nervous system. Since patients with hypertension should take antihypertensive drugs daily, the neuroprotective and antioxidant effects of antihypertensive drugs need to be investigated. In our previous study, the antioxidant effect of cilnidipine was identified (Lee et al., 2009). This finding also led us to investigate whether cilnidipine might protect neuronal cells from hypoxia.

Considering the fact that cilnidipine blocks both L- and N-type calcium channels and has an antioxidant effect against hydrogen peroxide, we investigated whether cilnidipine protects neuronal cells from hypoxia and aimed to identify the action mechanisms of cilnidipine in an *in vitro* model of ischemic stroke, focusing on the PI3K and ERK pathways.

2. Materials and methods

2.1. Materials

Cilnidipine was a generous gift from Boryung Co., Ltd. (South Korea). Before use, the cilnidipine was dissolved in dimethyl sulfoxide (DMSO), the final concentration of which was 1% (vol/vol) after dilution in culture medium, to yield the desired final concentrations. LY294002, a phosphatidylinositol 3-kinase (PI3K) inhibitor, and FR180204, a selective extracellular signal-related kinase (ERK) inhibitor, were purchased from Sigma (Saint Louis, MO, USA) and Santa Cruz Biotech (Santa Cruz, CA, USA), respectively.

2.2. Primary cultures and treatment of cortical neurons

All procedures using animals were consistent with Hanyang University's guidelines for the care and use of laboratory animals. We made every effort to minimize the number of animals used and any animal suffering. Each animal was utilized only once.

Primary cultures of cortical neurons were acquired from the cerebral cortices of fetal Sprague-Dawley rats (at 16 days gestation) (Noh et al., 2009). Briefly, rat embryos were decapitated, and their brains were isolated and put in a Petri dish half-filled with ice-cold Hank's balanced salt solution (HBSS; 137 mM NaCl, 5.4 mM KCl, 0.3 mM Na_2HPO_4, 0.4 mM KH_2PO_4, 5.6 mM glucose, and 2.5 mM Hepes; Gibco BRL, NY, USA). Single cells separated from whole cerebral cortices were seeded on 100 mm Corning dishes (5×10^6 cells/cm^2) coated with poly-L-lysine (Sigma, Saint Louis, MO, USA) or glass cover slips placed in 6- or 24-well Nunc plates (5×10^5, 2.5×10^6 cells/cm^2) and were suspended in 10% fetal bovine serum (FBS)/modified Eagle's medium (MEM). After 24 h, the medium was changed to the serum-free medium, neurobasal media (NBM) supplemented with B27. Cultures were kept at 37 °C under a humidified 5% CO_2 atmosphere. After being incubated for 6 days, the cells were fixed with 4% paraformaldehyde/PBS for anti-MAP-2 immunohistochemical staining. The neurite outgrowths were analyzed under a microscope. Only mature cultures (7 days *in vitro*) were used for experiments. The percentage of neuronal cells in the primary cultures was approximately 80% (Noh et al., 2009).

To determine the best hypoxic conditions for conducting our experiments, primary cultured cortical neurons were exposed to *in vitro* hypoxia (O_2 concentration = 0.1%) in an anaerobic chamber (Anaerobic System Model 1025, Forma Scientific, Marietta, OH, USA) equipped with a humidified, temperature-controlled incubator. After several hours of hypoxia, the cell viability was assessed 24 h after beginning the treatment using the lactate dehydrogenase (LDH) assay and trypan blue staining (TBS) (Lee et al., 2011).

To examine the effects of cilnidipine on the viability of primary cultured cortical neurons, we treated cortical neurons for 24 h with several concentrations of cilnidipine (0, 1, 2.5, 5, 10, 20, and 40 μM) and washed them several times with phosphate buffered saline (PBS). On the basis of our data indicating the effects of cilnidipine and hypoxia on neuronal cell viability, cortical neurons were treated with several concentrations of cilnidipine (0, 2.5, 5, 10, and 20 μM) and simultaneously exposed to hypoxic conditions for 8 h. The cells were then gently washed, and cell viability was evaluated 24, 48, and

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